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PRINCIPAL INVESTIGATOR: Hong Joo Kim
Dafna Bar-Sagi, Ph.D.

CONTRACTING ORGANIZATION: The Research Foundation of the State University of New York
Stony Brook, NY 11794-3362

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Hong Joo Kim
Dafna Bar-Sagi, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Research Foundation of the
State University of New York
Stony Brook, NY 11794-3362

E-Mail: hjkim@ic.sunysb.edu

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13. ABSTRACT (Maximum 200 Words)

Sprouty(Spry) was identified as a general inhibitor of receptor tyrosine kinases in *Drosophila* including FGFR and EGFR signaling. The mammalian genome contains four Spry genes (Spry1-4). Among these, Spry2 encodes a protein that displays the highest degree of homology with dSpry. Recently, it has been shown that hSpry1 and hSpry2 are downregulated in breast cancer, suggesting a role for Spry in modulating the growth properties of breast cancer cells. To investigate the role of hSpry2 in EGFR signaling, we have analyzed the effect of ectopic expression of hSpry2 on EGFR trafficking. hSpry2 resides in the trans-Golgi network (TGN) and is targeted to early endosomes via a process that is dependent on its ubiquitination. Upon reaching the early endosome, hSpry2 interacts with ubiquitin receptors that control the endosomal sorting of internalized growth factor receptors, thereby preventing further progression of EGFR along the endocytic sorting process. Our data indicate that hSpry2 may influence the signaling capacity of EGFR by altering its endocytic fate. The relevance of this mechanism to the link between EGFR activity and the development of breast cancer is under investigation.

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Introduction

Aberrant activation of epidermal growth factor receptors (EGFRs) has been implicated in the development and progression of human breast cancer (*1-3*). The amplification of EGFR gene and increased mRNA transcription has been reported in neoplastic human breast tissue (*4, 5*). Furthermore, elevated levels of epidermal growth factor (EGF) expression have been observed in most human breast cancer cell lines as well as in 83% of human breast cancer *in vivo* (*6*). Tumors that have alterations in EGFRs tend to be more aggressive, and are associated with factors that predict a poor clinical outcome (*5*), providing significant impetus for the development of targeted therapies. Therefore, understanding the function of these receptors in tumorigenesis and their regulatory mechanisms will be a key step in the development of target-specific cancer therapeutics.

Genetic studies have identified *Drosophila* Sprouty (dSpry) as a negative regulator of EGFR signaling. Four mammalian Spry proteins (Spry1-4) have been identified based on sequence similarities to dSpry (*7-9*). We and others have shown that mammalian Spry proteins interfere with EGFR trafficking. Since the signaling activity of EGFR is functionally linked to the trafficking of the receptor through different endocytic compartment, it is conceivable that the expression of Spry might have profound consequences on EGFR-dependent growth aberrations observed in breast cancer cells.

Results generated during last the funding period indicate that hSpry2 traffics from the trans-Golgi network (TGN) to the early endosome via a process that is dependent on its monoubiquitination. The current funding period has focused on an investigation of both the mechanism and functional significance of hSpry2 trafficking. As discussed below, progress has been made in characterizing the molecular basis of hSpry2 endosomal localization, and the effect of hSpry2 on EGFR trafficking.

BODY

During the last funding period (May 1, 2003 – April 30, 2004), we demonstrated that hSpry2 traffics from trans-Golgi to endosomes via a process that is dependent on its monoubiquitination. Wild-type hSpry2 localizes to the trans-Golgi and to endosomal compartments, whereas a ubiquitination-deficient mutant (hSpry2 ΔK, Figure 1A,B) is predominantly localized to the trans-Golgi apparatus (Figure 1C). The endosomal localization of hSpry2 ΔK is fully rescued by fusing a single ubiquitin moiety to the N-terminus of hSpry2 ΔK (Ub/hSpry2 ΔK) (Figure 1C-E), suggesting that mono-ubiquitination of hSpry2 regulates hSpry2 trafficking from the trans-Golgi to endosomes.

Ubiquitin is recognized by short protein domains such as ubiquitin interacting motif (UIM) and ubiquitin associated domain (UBA) (*10, 11*). The proteins that contain these ubiquitin binding domains, ubiquitin receptors, have been shown to regulate intracellular trafficking of ubiquitinated proteins (*12*). To investigate the mechanism by which hSpry2 trafficking is regulated, we sought to identify molecule(s) that can interact

with monoubiquitinated hSpry2. Among a number of ubiquitin receptors, we have focused on hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) because it localizes to the early endosome which is in the same location where monoubiquitinated hSpry2 is found (*13*). Hrs has a UIM motif and plays a central role in sorting ubiquitinated cargo to different destinations.

We found that hSpry2 interacts with both ectopically expressed and endogenous Hrs (Figure 2). In contrast to wild-type hSpry2, hSpry2 ΔK mutant shows a significantly reduced level of interaction with Hrs whereas Ub/hSpry2 ΔK displays an enhanced interaction with Hrs (data not shown). These data suggest that hSpry2 interacts with Hrs through its ubiquitin moiety and this interaction may stabilize the endosomal localization of hSpry2.

In order to further elucidate the physiological function of hSpry2 in breast cancer, we examined the effect of hSpry2 on EGFR signaling. It has been demonstrated that hSpry2 impedes EGFR endocytosis and trafficking by abrogating EGFR ubiquitination (*14-17*). The molecular mechanism has been suggested that EGF increases tyrosine phosphorylation of hSpry2, and augments an interaction between hSpry2 and the E3 ubiquitin ligase c-Cbl. This interaction inhibits ubiquitination of activated EGFR by c-Cbl and results in the hindrance of EGFR trafficking.

To define the spatial and temporal regulation of hSpry2 in EGFR trafficking, we analyzed the kinetics of EGFR trafficking in the presence of hSpry2. EGF stimulation initiated surface EGFR endocytosis. In 10 minutes, most of internalized EGFR colocalized with EEA1, an early endosomal marker, and gradually moved to the late endosomal compartment for the following time period, showing colocalization with Rab7, a late endosomal marker (Figure 3). Interestingly, the expression of hSpry2 delayed the movement of EGFR along the endocytic pathway. While 70% of internalized EGFR moves to the late endosomal compartment in control cells by 30 minutes, only 30% of EGFR is detected at the late endosomal compartment in hSpry2 expressing cells (Figure 3B,E). Seventy percent of EGFR remained at the early endosome in the presence of hSpry2 (Figure 3C,D). Surprisingly, the interference of EGFR trafficking by hSpry2 is not dependent on its ability to interact with c-Cbl, because the hSpry2 ΔCbl mutant, which does not associate with c-Cbl, also alters EGFR trafficking as effectively as wild-type hSpry2. These data suggest that hSpry2 might regulate EGFR signaling by interfering with its endocytic trafficking.

As described above, we have shown that hSpry2 interacts with Hrs. Recently, it has been reported that Hrs interacts with the tumor susceptibility gene 101 (Tsg101), another key component of endosomal sorting complexes. Furthermore this interaction is critical for the progression of activated EGFR from the early endosome to the late endosome (*18*). This phenotype is similar to that of hSpry2 expressing cells. Therefore, we investigated whether hSpry2 expression abrogates the interaction between Hrs and Tsg101 resulting in the impediment of EGFR trafficking. We found that ectopic expression of hSpry2 interferes with Tsg101 binding to Hrs in a dose dependent manner (Figure 4A). The interaction is regained when the level of Tsg101 expression is increased,

suggesting that hSpry2 and Tsg101 compete for Hrs binding (Figure 4B). hSpry2 ΔCbl is also able to interact with Hrs and disrupt the Hrs and Tsg101 interaction (Figure 4C, D), suggesting that this interaction is a Cbl binding domain (CBD) independent function. Taken together, these data suggest that hSpry2 interferes with EGFR trafficking by disrupting the interaction of Tsg101 with Hrs.

We are currently investigating the impact of this mechanism on the growth and survival of EGFR-positive breast cancer. Since hSpry2 is downregulated in most human breast cancer cells (*19*), the loss of a regulatory mechanism of EGFR signaling may impact the initiation and maintenance of breast tumors. To test this prediction, we are employing loss-of and gain-of function approaches to achieve two goals: (1) To investigate the effect of hSpry2 down-regulation in human non-tumorigenic breast epithelial cells (MCF-10A, AD074 and HME cells). (2) To elucidate the effect of hSpry2 overexpression in human breast tumor cells that have high EGFR profiles (BT-20, MDA-MB-231, Hs578T and EVSA-T cells).

To this end, we have utilized an RNA interference (RNAi) approache. We have generated small interference RNAs (siRNA) that specifically target *hSpry2*. The ability to downregulate hSpry2 proteins was successfully evaluated (data not shown). The effect of hSpry2 on alteration in proliferation and tumorigenesis of breast cells is under investigation.

Key Research Accomplishments

1. Identified Hrs as a ubiquitin receptor that recognizes monoubiquitinated hSpry2 and stabilizes its endosomal localization.
2. Demonstrated an effect of hSpry2 on delayed phase of EGFR trafficking from early endosome to late endosome.
3. Identified hSpry2 as a regulator for Hrs and Tsg101 interaction.
4. Characterized a novel mechanism by which hSpry2 interferes with EGFR signaling.

Reportable Outcomes

1. This research was presented in the American Society for Cell Biology 44th Annual Meeting. Abstract is attached in the appendices.
2. NIH RO1 grant is applied for based on work supported by this award with the title of "Functional analysis of the EGFR/Sprouty axis".

Conclusion

The output of a signal depends not only on activation of a particular set of signaling molecules but also on where and for how long the signal is emitted (*20-22*). Therefore, regulated internalization and movement of activated receptors through the

endocytic pathway is crucial for appropriate cellular outcomes. Work performed over the past year with the help of this fellowship has demonstrated that hSpry2 is a key molecule that has evolved to control EGFR signaling and is critical in maintaining an appropriate physiological signal. hSpry2 spatially and temporarily regulates the activated EGFR signals by modulating the endocytic pathway. Further investigation into the link of this mechanism to human breast tumorigenesis is in progress. This study is the first attempt to understand the mode of action of hSpry2 in the context of EGFR in human breast cancer. Therefore, it will help us understand the role of EGFR signaling in breast cancer development and provide new insights into therapeutic strategies for this disease.

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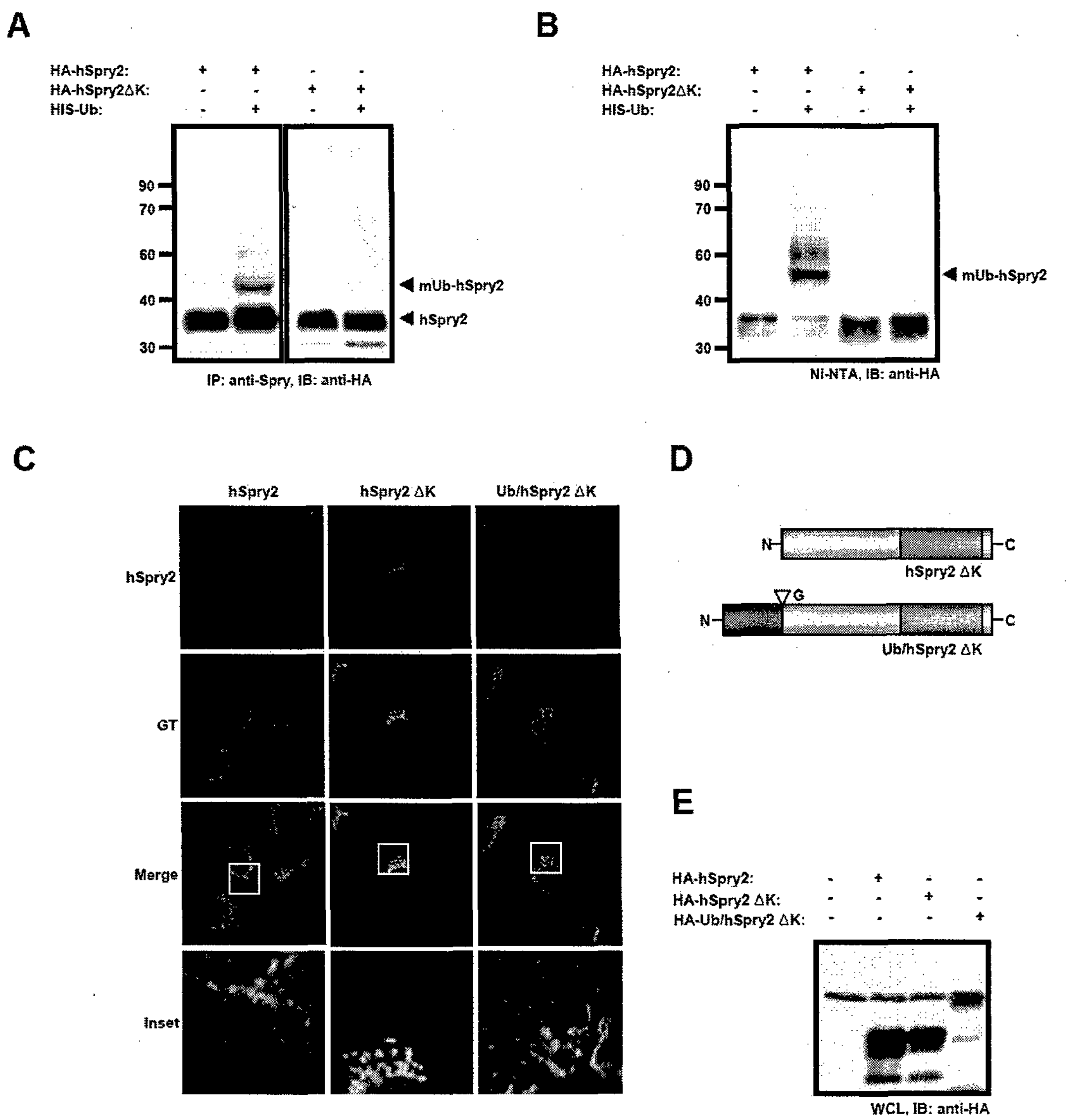

Figure 1. Monoubiquitination of hSpry2 regulates hSpry2 trafficking from trans-Golgi to endosomes. (A-B) A ubiquitination deficient mutant (hSpry2 ΔK) was generated by mutating every lysine residue on hSpry2. The ability of hSpry2 ΔK to be monoubiquitinated was tested by immunoprecipitation (A) and Ni-NTA affinity chromatography (B). (A) CHOK1 cells were transiently transfected with the vectors encoding HA-tagged Ubiquitin (HA-Ub) and HA-tagged hSpry2 construct as designated. Twenty-four hours post- transfection, whole cell lysates (WCL) were immunoprecipitated with anti-Spry antibodies, and immunoprecipitates were blotted with anti-HA antibodies. (B) CHOK1 cells were transfected with HIS-tagged ubiquitin (HIS-Ub) and HA-hSpry2 construct as designated. Cells were harvested in PBS followed by resuspension in buffer A (6M guanidine-HCl, 0.1M phosphate buffer, 10mM

imidazole) and lysed by sonication. Lysates were incubated for 3 hrs at room temperature with Ni-NTA resin. Resin was washed twice with buffer A, twice with buffer A/TI (1 volume buffer A, 3 volumes buffer TI) and once in buffer TI (25mM Tris-Cl, pH 6.8, 20mM imidazole), and proteins were eluted with SDS sample buffer supplemented with 200mM imidazole, and blotted with anti-HA antibodies. (C) MDCK cells were microinjected with GFP-tagged human beta 1,4 Galactosyltransferase (GT), a trans-Golgi marker, and HA-hSpry2 construct as designated. Six hours post-microinjection, cells were fixed with 3.7% paraformaldehyde for 1 hr at room temperature. Cells were then permeabilized for 3 min at room temperature with PBS containing 0.1% Triton X-100. Staining with primary antibodies (anti-Spry) was carried out for 1hr at 37°C in PBS containing 1% BSA. Cells were rinsed with PBS and staining with secondary antibodies was carried out for 1 hr at 37°C in PBS containing 1% BSA. Green: GT, Red: Spry, Blue: DAPI for nucleus. (D) Schematic representation Ub/hSpry2ΔK mutant. Ubiquitin is N-terminally fused to hSpry2ΔK mutant. To prevent polyubiquitin chains from being generated using lysine residues on ubiquitin, three lysine residues on ubiquitin (K29, K48 and K68) that can be conjugated by another ubiquitin were mutated to arginine residues. To block the cleavage of ubiquitin molecules from hSpry2 ΔK protein by a deubiquitinating enzyme, the last two glysine residues that serve for cleavage sites on ubiquitin were deleted. (E) Western blotting of hSpry2 proteins. CHOK1 cells were transfected with HA-hSpry2 construct as designated. WCL were immunoblotted with anti-HA antibodies.

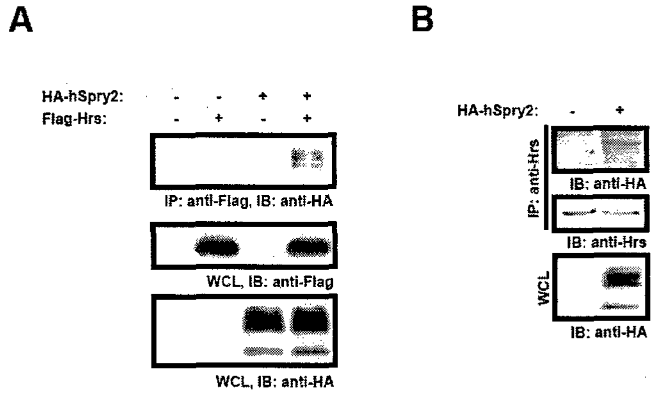

Figure 2. hSpry2 interacts with Hrs. (A) CHOK1 cells were transiently transfected with HA-hSpry2 and Flag- tagged Hrs (Flag-Hrs). Twenty-four hours post- transfection, whole cell lysates (WCL) were immunoprecipitated with anti-Flag antibodies, and immunoprecipitates were blotted with anti-HA antibodies. (B) CHOK1 cells were transiently transfected with HA-hSpry2. Twenty-four hours post-transfection, endogenous Hrs proteins were immunoprecipitated with anti-Hrs antibodies and immunoprecipitates were blotted with anti-Hrs and anti-HA antibodies.

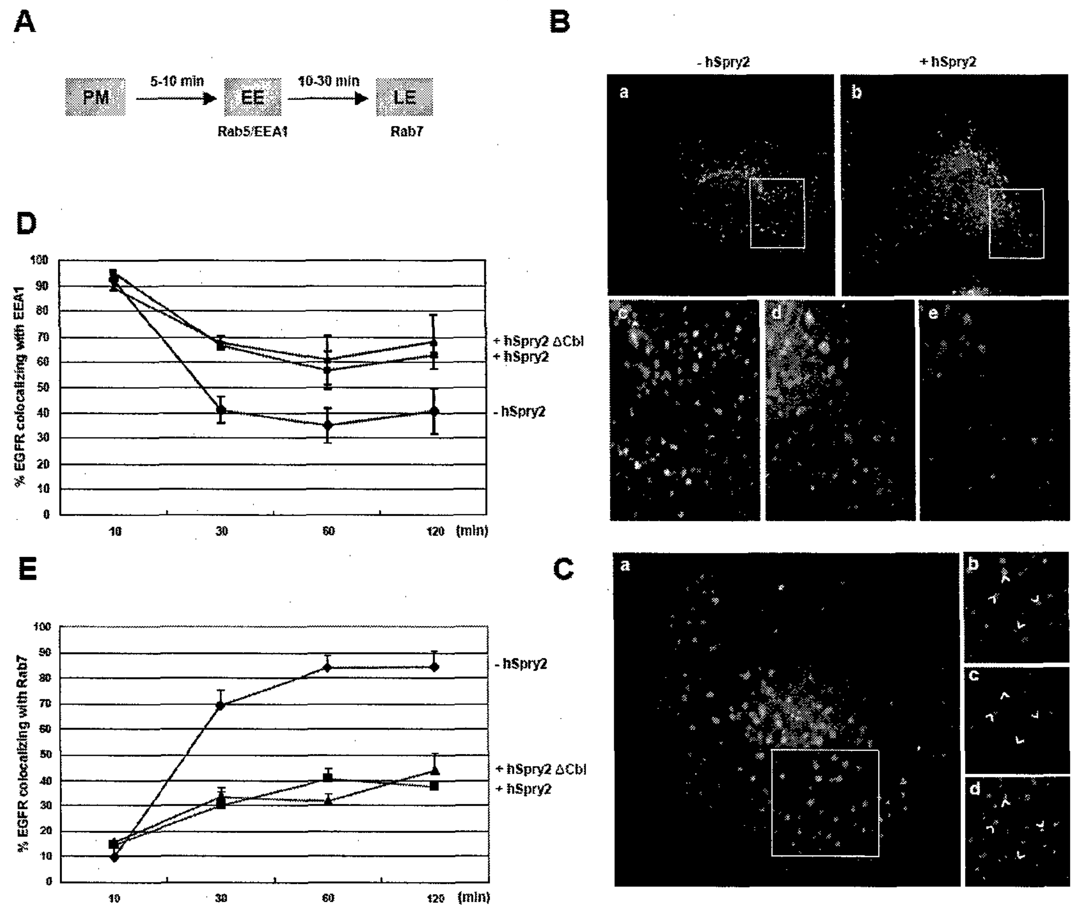

Figure 3. The effect of hSpry2 on EGFR trafficking. (A) Schematic representation of EGFR trafficking upon EGF stimulation. PM: Plasma membrane, EE: Early endosome, LE: Late endosome. (B) MDCK cells were serum-starved and microinjected with the vectors encoding EGFR, GFP-Rab7 and HA-hSpry2. Cells were stimulated with 100ng/ml EGF for 30 min and fixed with 3.7% paraformaldehyde for 1 hr at room temperature. Cells were then permeabilized for 3 min at room temperature with PBS containing 0.1% Triton X-100. Staining with primary antibodies was carried out for 1hr at 37°C in PBS containing 1% BSA. Cells were rinsed with PBS and staining with secondary antibodies was carried out for 1 hr at 37°C in PBS containing 1% BSA. Green: Rab7, Red: EGFR, Blue: Spry. (c) Inset from (a), (d and e) Inset from (b), (C) MDCK cells were serum-starved and microinjected with the vectors encoding EGFR, GFP-Rab5 and HA-hSpry2. Cells were stimulated with 100ng/ml EGF for 30 min and immunostained as described above. Green: Rab5, Red: EGFR, Blue: Spry. (b, c and d) Inset from (a). (D and E) Quantification of EGFR trafficking. In each time point, the percentage of EGFR colocalizing with endosomal marker out of total number of EGFR is plotted.

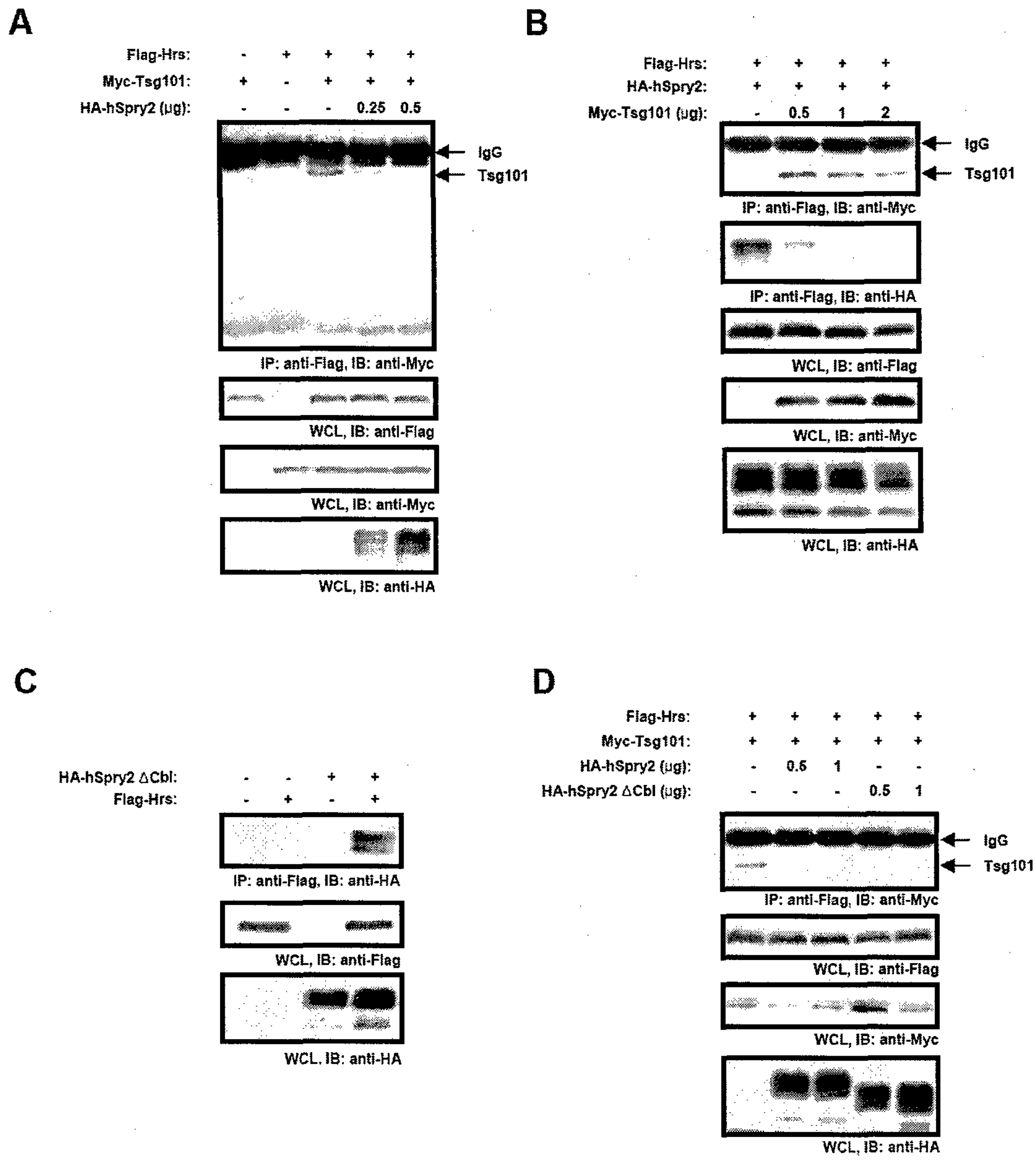

Figure 4. hSpry2 interferes with Hrs and Tsg101 interaction. CHOK1 cells were transiently transfected with the vectors encoding HA-hSpry2, HA-hSpry2 ΔCbl, Flag-Hrs and Myc-Tag101 as designated. Twenty-four hours post- transfection, whole cell lysates (WCL) were immunoprecipitated with anti-Flag antibodies, and immunoprecipitates were blotted with either anti-Myc antibodies (A, B and C) or anti-HA antibodies (B and C).

Curriculum Vitae

Name:	**Hong Joo Kim**	
Address:	(Work) SUNY at Stony Brook Life Science Building, Rm 225 100 Nicolls Rd Stony Brook, NY 11794	(Home) 11 Clematis street Port Jefferson Station, NY 11776-1819
E-mail:	hjkim@ic.sunysb.edu	
Phone:	(Work) (631) 632-9738	(Home) (631) 474-8352
Fax:	(Work) (631) 632-8891	

Education

9/00-present	Graduate student, Molecular and Cellular Biology program, State University of New York at Stony Brook, New York, USA.
3/97-2/99	M.S., Biochemistry and Molecular Biology, College of Medicine, Seoul National University, Seoul, Korea
3/93-2/97	B.S., Biology Education, College of Education, Seoul National University, Seoul, Korea

Research Experiences

9/00-present	State University of New York at Stony Brook, USA. Research Assistant in the laboratory of Dr. Dafna Bar-Sagi. Exploring the roles of hSpry2 on Ras/MAPK pathway.
3/99-6/00	ILCHUN Molecular Medicine Institute MRC, Seoul National University, Researcher in the laboratory of Dr. Jeong-Sun Seo. Construction of *clk-1* gene knockout mice.
3/98-2/99	Cancer Research Center, Seoul National University, Research Assistant in the laboratory of Dr. Jeong-Sun Seo Characterization of molecular structure and function of mouse *clk-1* gene
3/97-2/98	Transgenic Mice Center, Seoul National University Research Assistant in the laboratory of Dr. Jeong-Sun Seo Cloning and characterization of aging-related gene in mice.
12/95-2/96	Seoul National University, Undergraduate member in the laboratory of Dr. Tae-In Ahn

Awards

2004-Present	Department of Defense – Breast Cancer Research Program, Pre-doctoral Training Grant Awardee
2004	Sigma Xi Travel Award
1997-1999	Teaching Assistant Scholarship, Seoul National University
1997	Magna Cum Laude, Seoul National University
1993-1996	Sado Scholarship (*a merit-based scholarship*), Ministry of Education, Korea

Publication

Kim HJ and Bar-Sagi D (2003), Modulation of signaling by Sprouty: A developing Story, Nat Rev Mol Cell Biol. 5, 441-450

Presentation

1. **Kim HJ**, Taylor L, Bar-Sagi D (2004) hSpry2 impedes EGFR trafficking by a novel mechanism. The American Society for Cell Biology.
2. **Kim HJ**, Jura N, Da-Silva J, Bar-Sagi D (2003) Regulation of hSpry2 function by RTK signaling. Oncogene.

Appendices

hSpry2 Impedes EGFR Trafficking by a Novel Mechanism

H. Kim,[1] L. Taylor,[2] D. Bar-Sagi[2];
[1] Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY, [2] Microbiology and Molecular Genetics, Stony Brook University, Stony Brook, NY

Receptor tyrosine kinases (RTK) control a wide variety of cellular processes including cell proliferation, differentiation, migration and cell survival. The extent and duration of RTK signaling need to be precisely regulated to ensure proper cellular responses. Genetic studies have been identified *Drosophila* Sprouty (dSpry) as a negative regulator of RTK signaling. The mammalian genome contains four Spry genes (Spry1-4). Among these Spry2 encodes a protein that displays the highest degree of homology with dSpry. While investigating the role of human Spry2 (hSpry2) in the regulation of epidermal growth factor receptor (EGFR) signaling, we have uncovered an unexpected role for hSpry2 in modulating the endosomal fate of EGFR. hSpry2 traffics from the trans-Golgi network (TGN) to early endosomes via a process that is dependent on its ubiquitination. Accordingly, a ubiquitination-deficient hSpry2 mutant is retained in the TGN and this defect is rescued by fusing hSpry2 to a ubiquitin polypeptide. Upon reaching the early endosome, hSpry2 interacts via its ubiquitinated moiety with ubiquitin receptors that control the endosomal sorting of internalized growth factor receptors, thereby preventing further progression of EGFR along the endocytic sorting process. Taken together, our results reveal that hSpry2 ubiquitination serves as a sorting signal targeting hSpry2 from TGN to endocytic vesicles where it interferes with the delivery of EGFR to lysosomes by a novel mechanism.